DEHALOGENASE: THE FOLLOW-UP ENZYME AFTER MUSTARD OXIDATION

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ABSTRACT

Sulfur mustard (HD) has been used as a chemical warfare agent since 1917. Currently fielded M258A1 and M280 decontamination kits and prospective oxidative decontaminants convert HD to HD sulfoxide (HDSO). Although, the latter is not considered a vesicant, it is highly recalcitrant and its systemic toxicity, reportedly, is comparable to the agent. We found that live cells of a known hexachlorocyclohexane (lindane) metabolizing *Sphingomonas paucimobilis* bacterium could degrade HDSO. Cell free crude extract was obtained and found capable of HDSO enzymatic dehalogenation. Dehalogenase activity was monitored by determining chloride release using the Iwasaki colorimetric method. The reaction was also monitored by using GC-FPD and GC-ECD on derivatized samples. These results demonstrate the potential use of *S. paucimobilis* derived enzyme(s) in furthering the destruction of HD agent, thus providing significant contribution to the "Green" solution of the decontamination problem. This could be an important factor for the US and other nations in attempting to meet the requirements of 1993 Chemical Weapons Convention to destroy all chemical warfare agents within ten years of ratification (April 2007 for the US).

INTRODUCTION

The emphasis is to develop CW decontamination approaches that are more environmentally friendly than currently employed decontamination solutions, such as DS2. Bicarbonate/peroxide system (BAP) is one of the formulations proposed for this purpose (Wagner, 1999; Richardson, 1999). It has been shown that mustard sulfoxide (HDSO) is the sole product of sulfur mustard (HD) decontamination with BAP (Wagner, 1999; Drago, 1998). It has been reported that the use of other oxidative decontaminant systems with HD, including M258A1 and M280 kits for skin and individual equipment decontamination, also produces HDSO (Yang, 1992). Although the latter has been shown not to be a vesicant, its subcutaneous toxicity has been reported to be comparable to that of HD (Marshall, 1921; Groesbeck, 1923). In addition, HDSO is highly recalcitrant, reportedly resistant to hydrolysis even at 100°C (Helfrich, 1920).

We are interested in finding enzymes that are capable of hydrolytic removal of chlorine from HDSO. In this effort, we screened the microorganisms that have been reported to possess dehalogenase

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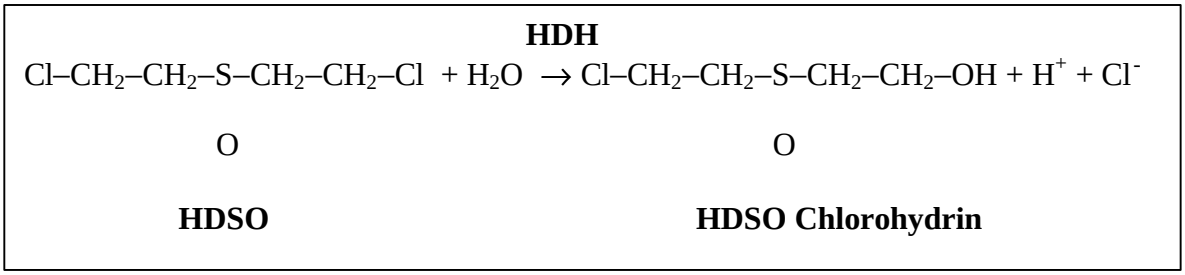
enzymes. A bacterial strain, *Sphingomonas paucimobilis* UT26 (formerly known as *Pseudomonas paucimobilis* UT26), was found to grow on hexachlorocyclohexane (HCH, or lindane) as a sole source of carbon (Senoo, 1989; Imai, 1989). Subsequently, the enzymes involved in HCH degradation had been purified (Nagata, 1993a, 1997) and the relevant genes in the metabolic pathway were cloned and sequenced (Imai, 1991; Nagata, 1993b, 1994; Miyauchi, 1998).

We obtained the *S. paucimobilis* UT26 strain and tested the live bacterial cells for HDSO dehalogenation activity. After ascertaining that the live cells were capable of removing chloride from HDSO, the organism was studied for the HDSO halidohydrolase (HDH) enzymatic activity (Scheme 1).



SCHEME 1. HDSO dehalogenation reaction by halidohydrolase (HDH).

MATERIALS AND METHODS

Organism and Growth Conditions: *S. paucimobilis* UT26 strain was kindly provided by Dr. Nagata. Unless otherwise indicated, the cultures were grown in a Luria broth (Nagata, 1999) supplemented with nalidixic acid containing (per liter): 3.3 g Bacto tryptone, 1.7 g yeast extract, 5 g sodium chloride, 25 mg nalidixic acid. To ascertain HDH activities of different bacterial crude extracts, one-liter Erlenmeyer flasks with 300 ml media inoculated with *S. paucimobilis* UT26 were routinely incubated at 30 °C for 40-70 hours on a recirculatory shaker at 200 rpm. For the enzyme purification, *S. paucimobilis* UT26 was cultured in a six 4-liter flasks at 150 rpm in a total of 10 liters of the medium. The cells were collected by centrifugation at 7,000×g for 30 minutes at room temperature, gently resuspended in a minimum amount of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.7 and spun again at 24,000×g for 20 minutes at 4°C. The supernatant was aspirated and the pellet was stored below -85°C. Unless otherwise indicated, subsequent procedures for the enzyme extraction and purification were conducted below 4°C. Also, all subsequent centrifugations were conducted at 24,000×g and enzyme fractions were stored below -85°C.

Enzyme Extraction: Frozen pellets of collected cells were resuspended in 20 mM HEPES buffer, pH 7.8 (3 ml per gram of wet weight). The cells were disrupted by passage through pre-chilled French Pressure cell (SLM-Aminco) at 16,000 psi three times. Crude cell extracts were obtained after the removal of cellular debris by centrifugation at 37,000×g for 30 min at 4 °C. In order to destroy heat labile enzymatic activities in a sample of supernatant, a small portion of the supernatant was boiled for five minutes and precipitates were removed by centrifugation at 13,000×g for 10 minutes.

Enzyme Purification on Ion-Exchange Column: Crude extracts were chromatographed on a DEAE-Sepharose Fast Flow (Amersham/Pharmacia Biotech Inc., Piscataway, NJ) anion-exchange column (25×150 mm). Before the sample application, the column was charged with two column volumes of the 2 M sodium acetate solution, and equilibrated with five column volumes of the 20 mM HEPES buffer, pH 7.8. After sample loading, the column was washed with four column volumes of the 20 mM HEPES buffer, pH 7.8, followed by the same amount of the 20 mM HEPES, 0.1 M sodium acetate,

buffer, pH 7.8 to elute loosely bound proteins. The enzyme was eluted with the 20 mM HEPES, 0.3 M sodium acetate, buffer, pH 7.8. Active fractions (60 ml) were pooled and frozen. The pool of the active fractions was defrosted and was diluted with 440 ml of the 20 mM HEPES buffer, pH 7.8, to be applied on the linear gradient anion-exchange column. The same DEAE-Sepharose Fast Flow anion-exchange column employed for the step gradient chromatography was used for the linear gradient chromatography. The column treatment, sample loading and pre-elution washes were conducted as above. Loosely bound proteins were again eluted with four column volumes of 20 mM HEPES, 0.1 M sodium acetate, buffer, pH 7.8. The enzyme was eluted by 2000 ml of a 0.1 to 0.27 M sodium acetate linear gradient in 20 mM HEPES buffer, pH 7.8. Active fractions (50 ml) were analyzed and stored frozen below -85 °C.

Molecular Weight Determination on Gel-filtration Column: The enzyme solution (0.2 ml) was applied on an HPLC (high-performance liquid chromatography)-GTi system (LKB) using GF-250 column (0.94 by 25 cm; DuPont) and eluted with the 20 mM HEPES, 0.1 M sodium acetate buffer, pH 7.8 at a flow rate of 1.0 ml/min.

Enzyme Assays: HDH enzymatic activity were routinely assayed in 20 mM HEPES buffer, pH 7.7 at 30°C or 37°C. Bis-Tris propane buffer (50mM) was substituted instead of HEPES buffer to study the pH optimum of HDH activity. Stock solution (50 mM) of mustard sulfoxide (HDSO) was added to the reaction medium to achieve the final concentration of 10 mM. At the appropriate times, the aliquots were withdrawn from the reaction medium for chloride concentration determination or were dried and derivatized for GC-FPD and GC-ECD analysis. One unit of HDH enzyme is defined as the catalyzing activity that released one μmole of chloride in one minute.

Chloride Determination: To determine chloride concentrations in the reaction medium we used the method of Iwasaki (Iwasaki, 1952) as follows: the reaction was stopped and the orange color was developed with the addition of 10% (v/v) 0.25 M $FeNH_4(SO_4)_2$ in 9 M HNO_3 and 10% (v/v) of 0.9 M $Hg(SCN)_2$ in ETOH. Precipitates were removed by centrifugation at 12,000×g for 2 min and the absorbance of the supernatant was read at 460 nm. It should be noted that the standard curve of the chloride although reproducible was not linear (Fig.1). Sigmastat application's polynomial regression analysis was used to find the coefficients of the polynomial expression that give the best fitting curve for the experimental data points of the standard curve. The derived polynomial function was used to ascertain the chloride concentrations from the absorbance of the sample.

Protein Determination: For protein determination, a protein dye binding method (Bradford, 1976) was used, with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

HDH activity in crude extract: We obtained the cell free crude extracts of the *S. paucimobilis* UT26 strain by homogenizing the cells with French Pressure cell and subsequent centrifugation of the homogenate. In order to find out whether the extract had HDH enzymatic activity, the native supernatant and the supernatant denatured by boiling was separately incubated in the reaction medium that contained HDSO (10 mM). A steady increase of chloride release from HDSO was observed in the incubation medium containing the supernatant, but not in the medium containing the boiled supernatant (Fig. 2). This indicates that the supernatant possessed HDSO dehalogenase enzyme that is denatured by boiling. The HDH catalytic activity of the extract was found to be 5.6 units per gram protein at 30.5 °C, at 10.6 millimolar HDSO concentration and pH of 7.7. HDSO degradation by crude extract was also confirmed by GC-FPD and GC-ECD analysis of derivatized samples (data not shown).

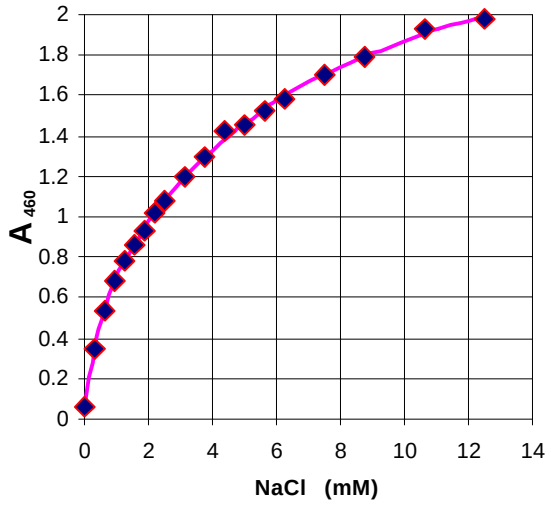


Figure 1. Chloride standard curve.

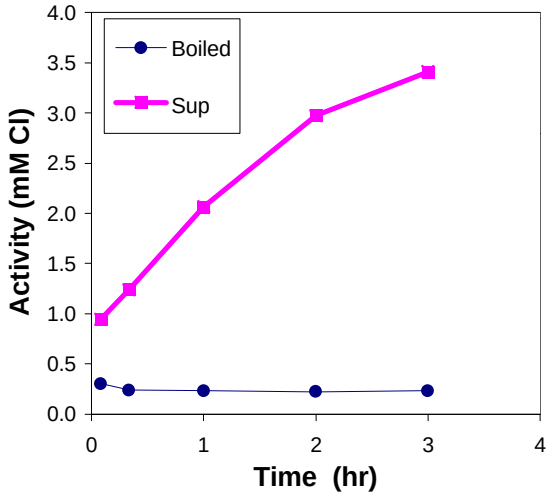


Figure 2. Boiling abolishes HDH activity.

Determining temperature and pH optimum: In order to find the temperature optimum of the HDH enzymatic activity, the experiments were conducted at temperatures varying from 25 °C to 30 °C at pH 7.7 (Fig. 3a). The maximum enzymatic activity was observed at 37 °C. The enzymatic activity at 30 °C was about 65 percent of the maximum and 45 percent at 25 °C. The enzymatic activity was nearly abolished at 49 °C (about 7.5 percent of the maximum). The initial experiments at 37 °C showed that the optimum enzymatic activity was above pH 7.5 (data not shown). Therefore, further studies were conducted in buffers with varying pH from 7.35 to 9.95 (Fig. 3b). We found that the HDH enzymatic activities increased with the increasing pH in the entire pH range that was tested. However, while HDSO was very stable at near neutral pH, the compound became unstable above pH 8.5 demonstrating increasingly higher spontaneous hydrolysis rate with increasing pH (Fig. 3b). Therefore, pH range of 8.0 to 8.5 was selected as the most suitable pH range for the analysis of HDH enzymatic activity.

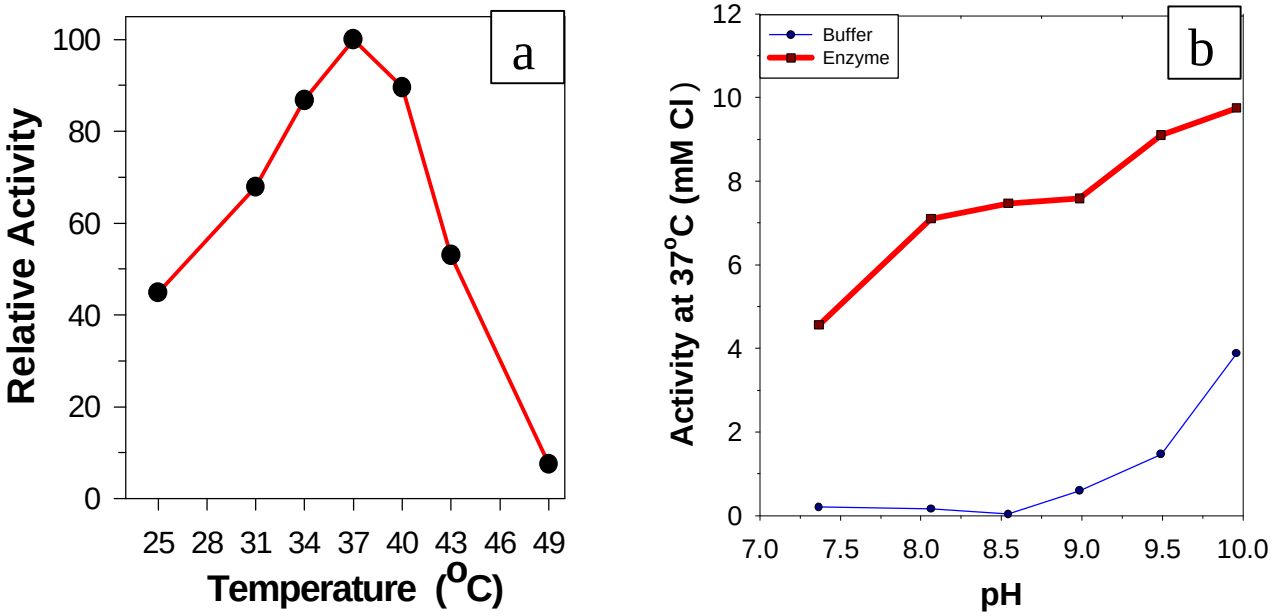


Figure 3. HDH activity versus (a) temperature and (b) pH.

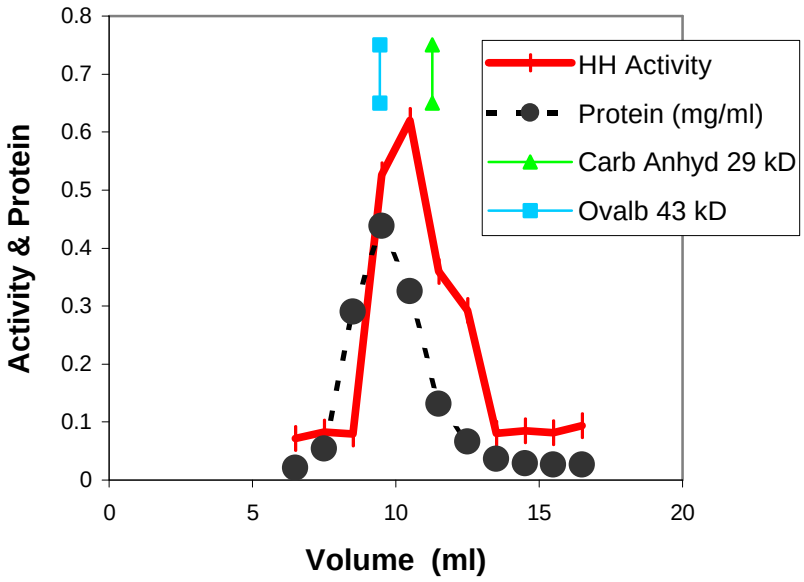


Figure 4. HDH gel-filtration on GF-250 column.

Chromatography: The gel-filtration HPLC chromatography was used to estimate the approximate size of HDH enzyme (Fig. 4). The maximum HDH enzymatic activity was eluted between the peaks of protein molecular weight standards of carbonic anhydrase (29 kD) and ovalbumin (43 kD). This result is consistent with earlier finding for LinB protein's gel-filtration elution profile and its deduced molecular weight of 33.1 kD (Nagata, 1993b, 1997). The HDH enzyme was partially purified on DEAE-Sepharose using the sodium acetate step gradient (Fig. 5), followed by the linear gradient of sodium acetate (data not shown). Further purification of the enzyme is in progress.

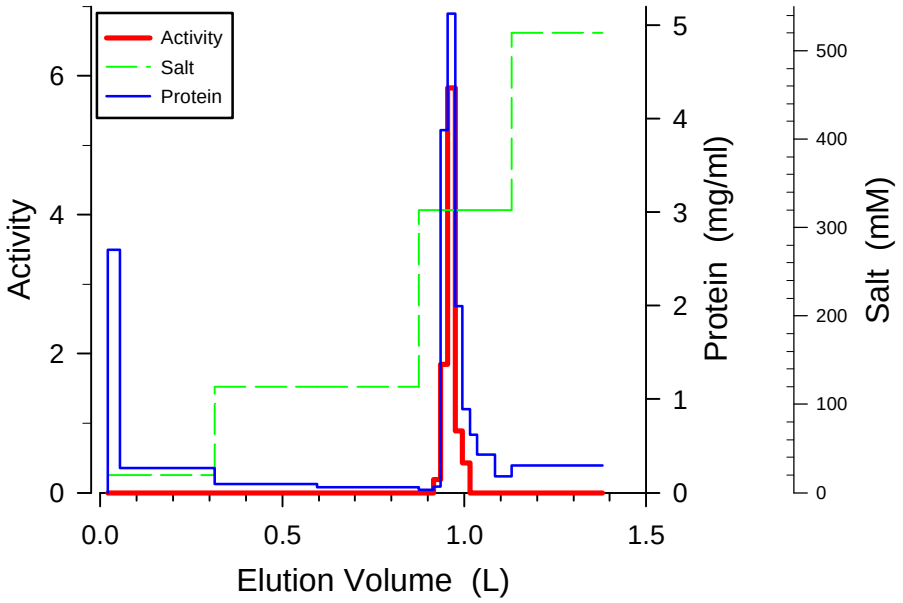


Figure 5. HDH purification on DEAE-Sepharose FF.

REFERENCES

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Drago, R.S.; Frank, K.M.; Wagner, W.G. and Yang, Y.-C. (1998) Catalytic activation of hydrogen peroxide – a green oxidant system. Proceedings of the 1997 U.S. Army Edgewood Research, Development and Engineering Center Scientific Conference on Chemical and Biological Defense Research. ERDEC-SP-063, pp 341-342.

Groesbeck, W.M. (1923) The toxicity for white mice, by injection, of certain compounds chemically related to mustard gas. EAMRD-16, pp.7-8. War Department, Chemical Warfare Service, Edgewood Arsenal, MD.

Helfrich, O.B. and Reid, E.E. (1920) Reactions and derivatives of ❍,❍'-dichloro-ethyl sulfide. *J. Am. Chem. Soc.* **42**, 1208-1232.

Imai, R.; Nagata Y.; Senoo, K; Wada, H.; Fukuda, M.; Takagi, M. and Yano, K. (1989) Dehydrochlorination of ❋-hexachlorocyclohexane (❋-BHC) by ❋-BHC assimilating *Pseudomonas paucimobilis*. *Agric. Biol. Chem.* **53**, 2015-2017.

Imai, R.; Nagata Y.; Fukuda, M.; Takagi, M. and Yano, K. (1991) Molecular cloning of a *Pseudomonas paucimobilis* gene encoding a 17-kilodalton polypeptide that eliminates HCI molecules from ❋-hexachlorocyclohexane. *J. Bacteriol.* **173**, 6811-6819.

Iwasaki, I.; Utsumi, S. and Ozawa, T. (1952) New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. *Bull. Chem. Soc. Japan* **25**, 226.

Marshall, E.K. Jr. and Williams, J.W. (1921) The toxicity and skin irritant effect of certain derivatives of dichloroethyl sulfide. *J. Pharmacol. Exp. Ther.* **16**,259-272.

Miyauchi, K.; Suh, S.-K.; Nagata, Y. and Takagi, M. (1998) Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of ❋-hexachlorocyclohexane by *Sphingomonas paucimobilis*. *J. Bacteriol.* **180**, 1354-1359.

Nagata, Y.; Hatta, T.; Imai, R.; Kimbara, K.; Fukuda, M; Yano, K. and Takagi, M. (1993a) Purification and characterization of ❋-hexachlorocyclohexane (❋-HCH) dehydrochlorinase (LinA) from *Pseudomonas paucimobilis*. *Biosci. Biotechnol. Biochem*. **57**, 1582-1583.

Nagata, Y.; Nariya, T.; Ohtomo, R; Fukuda, M; Yano, K. and Takagi, M. (1993b) Cloning and sequencing of a dehalogenase gene encoding an enzyme with hydrolase activity involved in the degradation of ❋-hexachlorocyclohexane in *Pseudomonas paucimobilis*. *J. Bacteriol.* **175**, 6403-6410.

Nagata, Y.; Ohtomo, R.; Miyauchi, K.; Fukuda, M; Yano, K. and Takagi, M. (1994) Cloning and sequencing of a 2,5-dichloro-2,5-cyclohexadiene-l,4-dial dehydrogenase gene involved in the degradation of ❋-hexachlorocyclohexane in *Pseudomonas paucimobilis*. *J. Bacteriol*. **176**, 3117-3125.

Nagata, Y.; Miyauchi, K.; Damborsky, J.; Manova, K.; Ansorgova, A. and Takagi M. (1997) Purification and characterization of haloalkane dehalogenase of a new substrate class from a ❋-hexachlorocyclohexane-degrading bacterium, *Sphingomonas paucimobilis* UT26. *Appl. Environ. Microbiol*. **63**, 3707-3710.

Nagata, Y.; Futamura, A.; Miyauchi, K. and Takagi, M. (1999) Two different types of dehalogenases, LinA and LinB involved in ❋-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26 are localized in the periplasmic space without molecular processing.. *J. Bacteriol*. **181**, 5409-5413

Richardson, D.E.; Yao, H.; Xu, C.; Drago, R.S.; Frank, K.M.; Wagner, W.G. and Yang, Y.-C. (1999) Kinetics and equilibrium formation of a weakly basic oxidant system for decontamination. Proceedings of the 1998 U.S. Army Edgewood Research, Development and Engineering Center Scientific Conference on Chemical and Biological Defense Research. ECBC-SP-004, pp 293-299.

Senoo, K. and Wada, H. (1989) Isolation and identification of an aerobic ❋-HCH decomposing bacterium from soil. *Soil Sci. Plant Nutr*. **35**, 79-87.

Wagner, W.G. and Yang, Y.-C. (1999) Baking soda, hydrogen peroxide, alcohol: the refreshing, universal decon for VX, GB and HD. Proceedings of the 1998 U.S. Army Edgewood Research, Development and Engineering Center Scientific Conference on Chemical and Biological Defense Research. ECBC-SP-004, pp 285-291.

Yang, Y.-C.; Baker, J.A. and Ward, J.R. (1992) Decontamination of chemical warfare agents. *Chem. Rev.* **92**, 1729-1743.